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Genetic Basis of Virulence in *Shigella* Species

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BRIEF HISTORICAL PERSPECTIVE

Bacillary dysentery was first differentiated from amoebic dysentery in 1887 and an etiologic agent, *Bacillus dysenteriae*, was isolated and described by Shiga in 1898. The subsequent painstaking process of epidemiological, physiological, and serological characterization of related dysentery bacilli culminated with the recommendations of the 1950 Congress of the International Association of Microbiologists Shigella Commission that *Shigella* be adopted as the generic name and that species subgroups be designated A (*Shigella dysenteriae*), B (*S. flexneri*), C (*S. boydii*), and D (*S. sonnei*). The next milestone was the characterization of the basic virulence mechanism of shigellosis. By the late 1950s, it had been shown that shigellae can infect the corneal epithelium of guinea pigs (this is the basis of the Serény test) and it was also known that virulent organisms can be grown intracellularly in cultured mammalian cells (43, 155). Nonetheless, it was the prevailing view as late as 1960 that shigellae cause disease by elaborating endotoxin while adhering to the surface of the intestinal epithelium (155). In 1964, however, it was conclusively demonstrated that *S. flexneri* causes disease by penetrating the intestinal mucosa (76, 147).

During the late 1960s and early 1970s the pathogenic mechanism of shigellosis was studied further, and the genetic basis of virulence was analyzed by constructing intergeneric hybrids of *S. flexneri* and *Escherichia coli*. Nonetheless, a puzzling result of the latter work was the finding that essentially the entire chromosome of *S. flexneri* could be transferred to *E. coli* without reconstituting the virulence phenotype of the donor. This enigma was resolved by the seminal work of P. J. Sansonetti and colleagues in the early 1980s showing that virulence in *Shigella* species is dependent upon a family of large plasmids (115, 118, 119). Since this discovery, genetic analysis carried out in several laboratories has shown that a number of plasmid genes are necessary for virulence, and multiple regulatory loci in the plasmid and in the chromosome have also been identified. Confusing genetic nomenclature has been an unfortunate by-product of this international research effort. This review will describe known chromosomal and plasmid virulence-associated loci in the context of the pathogenic mechanisms of shigellosis and will present an annotated summary of genetic nomenclature which may be of assistance in further reading of both contemporary and older *Shigella* literature.

OVERVIEW OF EPIDEMIOLOGY

Diarrheal diseases claim the lives of at least five million children per year in developing countries (109), and shigellosis or bacillary dysentery is responsible for approximately 10% of these deaths (135). *Shigella flexneri* is the predomi-

nant species in endemic areas, accounting for approximately 50% of culture-positive disease (6). *S. dysenteriae* 1, the agent of epidemic shigellosis, is responsible for extensive outbreaks in Central Africa, Southeast Asia, and the Indian subcontinent. *S. dysenteriae* 1 is also isolated from up to 30% of dysentery patients in endemic areas (6). In developing countries, shigellosis is most common in children less than 5 years old and is usually spread by the excreta of infected individuals either directly by the fecal-oral route or by contaminated food, flies, or water. Overcrowded conditions and water supplies that are inadequately protected from sewage contamination contribute to the high incidence of infection.

In developed countries, common-source outbreaks, usually involving *S. sonnei*, occur sporadically, and the source of such outbreaks is often uncooked food such as a salad that contains carbohydrates or proteins (9). Homosexual men are also at risk for direct transmission of *Shigella* infections, and recurrent shigellosis complicating human immunodeficiency virus infection can occur (10). Direct fecal-oral contamination can contribute to endemic shigellosis in institutional environments such as mental hospitals, day care centers, nursing homes, prisons, and outdoor gatherings. For example, a recent outbreak of *S. sonnei* among 12,700 attendees at an outdoor conference was characterized by an attack rate of greater than 50% (156). The epidemic strain was unusual for a North American isolate in that it was resistant to trimethoprim-sulfamethoxazole. It is interesting that 48 laboratory-confirmed cases of shigellosis due to this antibiotic-resistant strain were later reported in six states. None of these cases could be epidemiologically linked to the original outbreak.

OVERVIEW OF PATHOGENESIS

As illustrated by the epidemiological study discussed above, shigellosis is an unusually contagious infection. Under experimental conditions, ingestion of as few as 10 organisms can cause disease in 10% of North American volunteers, and ingestion of 500 organisms routinely causes disease in 50% of these volunteers (28). The reason(s) for the low 50% infective dose of *Shigella* species is not readily apparent, but the relative resistance of shigellae to stomach acid when compared with salmonellae or *E. coli* may facilitate the survival of small numbers of ingested organisms and provide the opportunity for organisms to infect the intestinal mucosa (44a). The clinical signs of shigellosis range from mild diarrhea to severe dysentery with frequent passage of bloody, mucoid, small-volume stools. Neurologic symptoms such as lethargy, confusion, severe headache, and convulsion are the most common extraintestinal manifestations of shigellosis (2). *Shigella* infections are usually self-limiting, but bacillary dysentery can be life-threatening in infants as a result of dehydration or chronic malnutrition (6, 109). Diarrheal disease is most often associated with *S. sonnei* infection, whereas *S. flexneri* and *S. dysenteriae* may have a prodrome of diarrhea but are usually characterized by dysentery (135).

Although the molecular basis of shigellosis is complex, the initial step in pathogenesis is clearly bacterial invasion or penetration of the colonic mucosa (76, 83, 138, 147). The resulting focus of *Shigella* infection is characterized by degeneration of the epithelium and by an acute inflammatory colitis in the lamina propria. Ultimately, desquamation and ulceration of the mucosa cause leakage of blood, inflammatory elements, and mucus into the intestinal lumen. Under these conditions the absorption of water by the colon is inhibited and the volume of stool is dependent upon the ileocecal flow (13). As a result, the patient will pass frequent, scanty, dysenteric stools.

In contrast to dysentery, the pathologic basis of *Shigella* diarrhea is unclear. In rhesus monkeys, passage of shigellae through the small intestine is a prerequisite for diarrheal symptoms (110), but in humans, this organ is not usually colonized by large numbers of organisms (83). *S. dysenteriae* 1 is unique among *Shigella* species in that high levels of both enterotoxic and cytotoxic Shiga toxin are present in culture filtrates (29). *S. sonnei* and *S. flexneri* express detectable levels of cell-bound cytotoxin(s), but this toxic activity is much lower than the cytotoxic activity of the *S. dysenteriae* 1 Shiga toxin (2, 96, 107). Weak enterotoxic activity has recently been detected in cultural supernatants from shigellalike enteroinvasive *E. coli* (EIEC) strains, and preliminary data suggest that analogous enterotoxic activity is present in *S. flexneri* culture supernatants (32).

Although uncharacterized *Shigella* enterotoxin(s) may play a role in ileal fluid secretion, the fact remains that diarrhea is most common in patients suffering extensive *Shigella* colitis in the transverse colon or cecum (133). Net secretion of water, as well as impaired colonic absorption, has been observed in these individuals (13). Since indomethacin (an inhibitor of prostaglandin synthesis) decreases fluid secretion in ligated rabbit ileal loops injected with invasive *S. flexneri* (45), it is possible that prostaglandins elicited by the inflammatory response to bacterial invasion contribute to diarrhea in patients with *Shigella* colitis (129).

In addition to the acute symptoms of shigellosis, chronic sequelae have been associated with these infections. For example, infections with *S. flexneri* are associated with reactive arthritis or Reiter syndrome. A strong predisposition for this sequela is found in individuals who express the HLA-B27 histocompatibility antigen. Monoclonal antibody (MAb) raised against B27 epitopes cross-reacts with 36- and 23-kDa proteins of *S. flexneri* (12), and MAb raised against a 36-kDa *S. flexneri* outer membrane protein cross-reacts with lymphocytes expressing HLA-B27 (157). Since these reactive proteins are present in many gram-negative bacterial species, it has been suggested that additional bacterial characteristics such as invasiveness are required to induce an autoimmune response in HLA-B27-expressing individuals (157). In addition, a survey of the plasmids of arthritogenic and nonarthritogenic strains of *S. flexneri* indicates that a 2-MDa plasmid is present only in the former strains. Sequencing of this plasmid reveals an open reading frame (ORF) encoding a stretch of five amino acids that duplicate residues 71 to 75 of the polymorphic region of the α1 domain of HLA-B27 (134), but expression of this epitope by shigellae has not been demonstrated. Although demonstration of immunodeterminants common to both HLA-B27 and *S. flexneri* lends support to the concept of molecular mimicry in reactive arthritis, current data do not definitively characterize this sequela.

Hemolytic-uremic syndrome, a rare sequela characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure, has been observed in children infected with *S. dysenteriae* 1. This complication has been attributed to circulating immune complexes of lipopolysaccharide (LPS) endotoxin associated with severe colitis (74). However, epidemiological data suggest that Shiga toxin produced by *S. dysenteriae* 1 (or by *E. coli* strains that are agents of hemorrhagic colitis) could be the underlying cause of hemolytic-uremic syndrome (85).

LABORATORY MODELS OF PATHOGENESIS

As is the case with most pathogenic microorganisms, genetic analysis of virulence in *Shigella* species is dependent upon laboratory models that mimic the natural infection. Study of the pathogenic mechanism of shigellosis has been complicated by the innate resistance of most animals to oral infection by shigellae. However, animal models can reproduce some aspects of human shigellosis. For example, oral challenge of starved, opiated guinea pigs with *S. flexneri* 2a first established the invasive nature of *Shigella* infections (76), and oral challenge of rhesus monkeys, which are susceptible to naturally acquired *Shigella* infections, confirmed the invasive pathogenic mechanism (138, 147). Injection of shigellae into ligated rabbit ileal loops has been used as a model of bacterial invasion which elicits fluid secretion (45, 147). Complementing these animal models of intestinal shigellosis is the Serény test, which assesses the ability of shigellae to invade the corneal epithelium and to elicit keratoconjunctivitis in the eyes of rabbits, guinea pig, or mice. Tissue culture monolayers are also invaded by shigellae (43, 49, 52, 76), and the spread of shigellae to contiguous cells within the monolayer can be documented by the cytopathic effect of this intercellular spread, i.e., plaque formation (99, 105, 123).

CHROMOSOMAL GENES ASSOCIATED WITH VIRULENCE OF SHIGELLAE

S. flexneri and *E. coli* share a common system of mating polarities and many common gene linkage patterns (86). In fact, the two species are so closely related they cannot be distinguished on the basis of polynucleotide hybridization (11). Nonetheless, conjugal transfer of chromosomal material from Hfr *E. coli* K-12 to *S. flexneri* 2a can result in decreased virulence (31, 35, 36). For example, *S. flexneri* 2a transconjugants that had acquired the Xyl marker (80 min) after matings with an *E. coli* K-12 Hfr strain were the first intergeneric hybrids demonstrating decreased lethality in the orally challenged guinea pig model (31, 36). Since these hybrids had consistently incorporated the unselected Rha (88 min) and Arg (90 min) markers in addition to Xyl, virulence determinants located within a 10-min segment of the *Shigella* chromosome were potentially displaced in these matings. In converse matings, transfer of chromosomal markers from Hfr *S. flexneri* 2a into an *E. coli* K-12 recipient reconstitutes certain aspects of the virulent phenotype (105, 116). For example, conjugal transfer of the Arg, His, Pro, and *purE* chromosomal regions into a K-12 recipient that has acquired the *S. flexneri* 5 virulence plasmid reconstitutes an invasive hybrid that can elicit fluid in rabbit ileal loops and evoke a positive Serény test (116).

It is not clear exactly how much of the Hfr genome was transferred in these classic genetic recombination studies, and the molecular basis of the altered virulence phenotype is not completely understood. Nonetheless, conjugal matings, transposon mutagenesis, site-directed mutagenesis, and construction of cloned gene libraries have allowed the identification of a number of virulence-associated loci on the *Shigella* chromosome (Table 1). The products of these loci can be generally classified as (i) determinants that affect the ability of shigellae to survive in the intestinal tract or in the mucosal tissues, e.g., siderophores, somatic antigen, and superoxide dismutase; (ii) cytotoxins that contribute to the severity of disease; and (iii) regulatory factors that affect the expression of plasmid virulence genes.

Siderophores, Somatic Antigens, Superoxide Dismutase, and Cytotoxins

Aerobactin (*iucABCD* and *iutA*). Conjugal matings of an *S. flexneri* 2a Hfr strain with an *E. coli* K-12 recipient have revealed that the *xyl-rha* region of the donor contains the *iucABCD* locus encoding the hydroxamate aerobactin siderophore and the *iutA* gene that encodes a 76-kDa receptor protein (46). In *E. coli* these loci are carried on the pColV plasmid, but in *S. flexneri* they are linked to the *tnaA* gene located at 83 min on the *E. coli* chromosomal map (26). Biosynthetic and transport genes for aerobactin have been found in all *Shigella* species except *S. dysenteriae* type 1, but some strains of *S. sonnei* do not express these genes (77). The last two species usually express the enterobactin siderophore that maps at 13 min on the *E. coli* chromosome (3). Although *S. flexneri* strains also carry enterobactin genes, the presence of both an IS*1* element and a deletion mutation within this locus precludes expression of the siderophore (126).

Transposon mutagenesis of the aerobactin *iuc* locus in *S. flexneri* has allowed construction of aerobactin-negative mutants that were tested for growth in HeLa cells and in various animal models (78, 95). The ability of these mutants to invade and multiply within HeLa cells is similar to that of wild-type strains, and *iuc* mutants are also able to multiply in a lysate of HeLa cells. These data indicate that an adequate pool of iron is available under the reducing conditions prevalent in the mammalian cell cytoplasm or that heme-iron is sufficient to satisfy the requirements for intracellular bacterial growth in the absence of siderophore expression (106).

Although the tissue culture model fails to differentiate siderophore phenotypes, subtle differences in the virulence of aerobactin mutants can be detected in animal models. With a relatively small inoculum (1×10^7 cells), for example, an *iuc* mutant produces a delayed-positive Serény test (95). This inoculum also elicits minimal mucosal inflammation and fluid accumulation in rabbit ileal loops. When the inoculum is increased 10- to 100-fold, however, the *iuc* mutant is nearly as virulent as the parent strain. Thus, it appears that aerobactin facilitates the multiplication of shigellae within the tissues, but attenuation associated with the loss of siderophore expression can be overcome if the initial challenge includes large numbers of organisms.

Group-specific somatic antigen. A critical role for the LPS somatic antigen in pathogenesis can be inferred from the avirulence of rough shigellae in animal models such as the Serény test (103). Rough strains of *S. flexneri* retain the ability to invade tissue culture cells (103), and these strains multiply within the cells. However, rough strains do not spread to contiguous host cells or form plaques in tissue culture monolayers (102a). The latter observation suggests that physical characteristics imparted by a smooth LPS may affect the interaction of shigellae with the host cell cytoskeleton. In addition to defective intercellular spread, rough strains with exposed lipid A are probably more susceptible to nonspecific host defense mechanisms such as the LPS-binding protein (142). This bifunctional plasma protein specifically cross-links bacterial lipid A to a receptor on the mononuclear phagocytes and stimulates synthesis of tumor necrosis factor alpha, interleukin-1, and interleukin-6 lymphokines. Intrinsic biochemical characteristics of the rhamnose-rich *S. flexneri* group 3,4 antigen may also be necessary for pathogenicity since intergeneric *S. flexneri* hybrids expressing the chemically similar *E. coli* O25 antigen are 90%

TABLE 1. Chromosomal loci associated with virulence in *S. flexneri*

Locus	Linkage[a]	Map units (min)[b]	Virulence phenotype of mutants	Regulatory or effector function	Reference(s)
T-locus	*pro-lac*	7	Y variants expressing only group 3,4 somatic antigen may exhibit decreased virulence	Integration site for incorporation of lysogenic phage encoding type-specific somatic antigen	10, 34, 131, 132, 140
kcpA	*purE*	12	Serény negative with limited intracellular and intercellular bacterial spread in tissue culture monolayers	Positive regulation of plasmid gene *virG* (*icsA*)	62, 79, 87, 105, 158
virR[c]	*galU*	27	Invasive in tissue culture when grown at 30°C	Repression of plasmid invasion loci, e.g., *ipaABCD* in response to temperature	27, 44, 59, 62, 91
stx[d]	*pyrF*	28	Decrease in vascular damage in the colonic epithelium of orally challenged monkeys	Synthesis of Shiga toxin	67, 75, 96, 127, 136
rfb (allele)	*hisA* to *hisI*	44	Serény negative, decreased intercellular spread in infected tissue culture monolayers	Synthesis of group-specific somatic antigen	34, 42, 51, 102a, 116
ompR-envZ	*malA*	75	Decreased invasion in tissue culture; Serény negative	Induction of plasmid invasion loci, e.g., *ipaABCD*, in response to high osmolarity in colon	7, 38
rfa (allele)	*mtl*	81	Delayed or negative Serény test, decreased intercellular spread in infected tissue culture monolayers	Synthesis of somatic antigen basal core	102a
iucABCD-iutA	*tnaA*	83	Delayed Serény reaction, decreased mortality in orally challenged guinea pig; decreased histopathology and fluid accumulation in ligated rabbit ileal loop; loss of virulence in orally challenged monkey model	Synthesis of aerobactin and 76-kDa aerobactin receptor protein	25, 46, 77, 78, 95, 106
sodB	Unknown	Unknown	Sensitivity to oxygen-dependent killing by phagocytes; Serény negative; greatly decreased histopathology in ligated rabbit ileal loop	Superoxide dismutase inactivates superoxide radicals produced by respiratory burst in phagocytes	40

[a] *purE*, purine; *pyrE*, pyrimidine; *hisA* to *hisI*, histidine; *malA*, maltose; *mtl*, mannitol; *tnaA*, thymine.
[b] Map units as determined by reported time of entry in interrupted-conjugation experiments and by cotransduction of the linked genes in *E. coli* K-12 (3).
[c] The *virR* locus may be a homolog of the *E. coli osmZ* (*bglY*, *cur*, *pilG*, *drdX*) locus that affects DNA supercoiling.
[d] The *stx* gene is present only in the chromosome of *S. dysenteriae* 1.

Serény positive, whereas other hybrids expressing the chemically divergent *E. coli* O8 antigen are uniformly avirulent (42). *S. flexneri* somatic antigen facilitates adhesion to guinea pig intestinal mucus in vitro (66), and this characteristic may also aid in colonization of the colonic epithelium.

Since loci controlling synthesis of the serogroup B somatic antigen integrate into the *mtl* region (80 min) and *his* region (44 min) of the chromosome of *E. coli* K-12 recipients which have been mated with *S. flexneri* Hfr (34, 116), the *Shigella* LPS biosynthetic loci are alleles of the *mtl*-linked *rfa* locus and *his*-linked *rfb* locus of *E. coli*. The *rfa* locus is necessary for the synthesis of the LPS basal core region, and in *S. flexneri* this process proceeds from 2-keto-3-deoxyoctonate-lipid A by the sequential addition of L-glycero-*D-manno*-heptose phosphate, D-glucose, D galactose, and *N*-acetyl-D-glucosamine (reviewed in reference 151). A Tn*5* insertion in the *rfa* locus that increases the mobility of the core constituent of smooth *S. flexneri* 2a LPS results in a mutant exhibiting both delayed plaque formation and delayed Serény reactions (102a). This observation suggests that the structure of the LPS core affects the virulence of shigellae even when O repeat units are attached. Changes in the composition of the LPS core of *E. coli* K-12 have pleotropic effects on the expression of outer membrane proteins, such as the OmpC and OmpF porins (108). In addition, the trimerization of OmpA and OmpF in the outer membrane and the conformation of other outer membrane proteins is profoundly affected by the detergent characteristics of LPS core-lipid A in the bacterial membrane (108). Since OmpC and OmpF expression is necessary for normal growth of *S. flexneri* within tissue culture cells (7), it is possible that changes in core composition affect the virulence of shigellae by indirectly influencing the expression or conformation of outer membrane proteins.

The *rfb* locus encodes rhamnose synthetase, rhamnose transferase, and *N*-acetylglucosamine transferase, which are required for synthesis of the *N*-acetylglucosamine-rhamnose-rhamnose-rhamnose O repeat unit of the group 3,4 or Y variant antigen of *S. flexneri* serotypes 1a, 2a, and 5a (131). In serotypes 4a, 3a, and 5b the primary chain structure has different sugar linkages, and this structure has the serologically distinct group 7,8 or X variant antigenic specificity. The *S. flexneri* serotype antigens consist of secondary side chains of α-glucosyl or O-acetylated α-glucosyl residues attached to the primary structure. The completed individual repeat units are transferred to an antigen carrier lipid and polymerized by

the product of an unmapped polymerase gene (*rfc*). Finally, the completed O-specific side chain is attached to the basal core structure by translocase activities encoded by genes of both the *rfa* and the *rfb* loci (132).

Many of the *S. flexneri* serotype specificities are under the genetic control of temperate bacteriophages (131) that use the Y somatic antigen as a receptor for adsorption (41). These lysogenic phages integrate into the *Shigella* chromosome at the Type locus or T-locus (126) that is linked to the *pro* locus (6 min) and to the *lac* locus (8 min). Expression of type-specific antigens I, II, IV, V, and 7,8 can be eliminated by selection for the Lac marker in conjugal matings with an *E. coli* Hfr strain (86, 140), and the type II specificity can be transferred to *E. coli* by selection for the Pro marker (34). The T-locus controls the determinants in which glucose is the immunodominant sugar, whereas the converting phages which encode the type III and type IV antigen (*O*-acetylglucose) map elsewhere on the chromosome (131). Since Y variants of *S. flexneri* can cause disease (10), the T-locus is not technically a virulence locus. Nonetheless, immunity induced by *Shigella* infections is serotype specific (37, 39), and these antigens are apparently important protective immunogens.

In contrast to *S. flexneri*, *S. dysenteriae* and *S. sonnei* require plasmid-encoded enzymes for somatic antigen biosynthesis. The galactose transferase gene (*rfp*) is located on a 9-kb plasmid in *S. dysenteriae* (153, 154) (Table 2). In addition to this plasmid, His-linked genes from *S. dysenteriae* are necessary for core and repeat unit assembly in an *E. coli* K-12 hybrid (51). The amino sugar disaccharide repeat unit of the form I O antigen of *S. sonnei* is synthesized by genes located on the 180-kb virulence plasmid of this species (73, 118) (Table 2). The form I antigen locus has recently been cloned and characterized as a 12.6-kb DNA sequence consisting of four gene clusters. Products of 48, 42, 39, and 23 kDa are expressed by two of these gene clusters or operons, but specific enzymatic activities have not yet been assigned to the proteins. Since group D antigen was expressed in *E. coli* HB101 when the form I locus was cloned into this genetic background, *E. coli rfa* and *rfb* genes apparently support the biosynthesis of the *S. sonnei* O-specific antigen (158a).

Superoxide dismutase (*sodB*). Allelic exchange of the unmapped *S. flexneri* superoxide dismutase gene has been accomplished by P1 transduction of a *sodB-kan* mutant gene from *E. coli* K-12 (40). The resulting *sodB Shigella* mutant is extremely sensitive to oxygen stress and to killing by mouse peritoneal macrophages or human polymorphonuclear leukocytes. In contrast, 5 to 10% of the wild-type *S. flexneri* parent cells survive within these phagocytic cells. Since the *sodB* mutant causes relatively little histopathological damage to the mucosa of ligated rabbit ileal loops, the resistance of *sodB*$^+$ shigellae to cell-mediated bactericial activity is apparently a decisive factor in the genesis of *Shigella* colitis.

Shiga toxin (*stx*). *S. dysenteriae* 1 is unique among *Shigella* species in the synthesis of a potent cytotoxin designated Shiga toxin. This toxin binds to Galα1-4Galβ (galabiose) glycolipid receptors (84) and inhibits mammalian protein synthesis by cleaving the N-glycosidic bond at adenine 4324 in 28S rRNA. Therefore, the toxic mechanism is identical to that of the plant toxin ricin (30, 67). Unconcentrated culture filtrates of *S. dysenteriae* 1 also have enterotoxigenic activity that elicits fluid accumulation when injected into ligated rabbit ileal loops (29, 70).

Mutagenesis of the Shiga toxin A subunit gene by allelic exchange has allowed the construction of isogenic Tox$^-$ and Tox$^+$ *S. dysenteriae* 1 strains (33). In orally challenged monkeys, the Tox$^-$ strain evokes diarrhea and mucoid stools with pus, but there is little blood in the dysenteric stools. Histological analysis indicates that infections with the Tox$^-$ mutant are characterized by decreased capillary destruction in the colonic mucosa. Since a direct cytotoxic effect can be demonstrated on cultured human vascular endothelial cells in vitro (101), the vascular damage in the colonic mucosa of monkeys challenged with the Tox$^+$ strain is probably a cytotoxic manifestation of Shiga toxin.

Expression of Shiga toxin can be transferred to *E. coli* K-12 by a chromosomal region that integrates with *trp-pyrF* during matings with an *S. dysenteriae* 1 Hfr strain, and the toxigenic phenotype is cotransduced with *pyrF* (127). Thus, the approximate map location of the toxin locus (*stx*) is 28 min. Experiments with probes detecting the structural-gene sequences of Shiga toxin have confirmed that this locus is the toxin structural gene (96). On the basis of the sequences of the cloned *stxA* and *stxB* genes, the molecular masses calculated for the processed A and B subunits are 32,225 and 7,691 kDa, respectively (75, 136). The sequence of *S. dysenteriae* 1 Shiga toxin is identical to that of the Shiga-like toxin (SLT-I) of *E. coli* except for a threonine at position 45 of the A subunit that is replaced with a serine in SLT-I. Both *stxA* and *stxB* are present on a single transcriptional unit (136), but holotoxin consists of one A subunit and five or six B subunits. Although monocystronic mRNA transcribing only *stxB* has been detected in Northern (RNA) blots (75), the smaller RNA fragment could represent breakdown products. Since results of β-galactosidase fusions with the A- and B-subunit operons of SLT-I have recently indicated that the subunits are transcribed at approximately a 1:1 ratio, the biosynthetic mechanism underlying the 1:5 stoichiometry of the holotoxin remains a matter of conjecture (11a). The polycystronic *stx* operator region contains the binding site for the Fur protein, and this accounts for the negative effect of high iron levels on the expression of Shiga toxin. Multiple copies of the *stx* locus have been detected, and a flanking sequence that is nearly homologous to the IS*600* insertion element may be responsible for gene amplification (75).

Although the structural genes for Shiga toxin can be detected only in *S. dysenteriae* 1 by colony blot hybridization with an SLT-1 probe (97), cytotoxic activity that may be neutralized by rabbit antiserum raised against Shiga toxin is present in some cultures of *S. flexneri* 2a (96, 100, 107) and *S. sonnei* (96, 107) and in *S. dysenteriae* 1 strains that have suffered a deletion in the *stx* locus (96). Although the cytotoxic activity of cell extracts from these strains is 10^3- to 10^4-fold lower than that of extracts of a fully toxigenic *S. dysenteriae* 1 strain, infections with cytotoxic strains are characterized by higher incidences of fever and occult blood in the stools (107). Cytotoxic activity that may be neutralized by Shiga antitoxin can also be detected in extracts of *E. coli* K-12 strains that have acquired the *argE* gene cluster from the *S. flexneri* chromosome (141), and the ability of invasive *E. coli-S. flexneri* hybrids to evoke fluid accumulation when injected into ligated rabbit ileal loops is associated with the incorporation of the Arg or Mtl chromosomal markers from an *S. flexneri* 2a Hfr strain (116). The putative *arg*-linked cytotoxin-enterotoxin locus has been provisionally named *flu* (fluid accumulation) (159). Cytotoxic activity that cannot be neutralized by rabbit antiserum raised against Shiga toxin has also been found in *S. flexneri* and *S. sonnei* strains, and these non-SLTs have been associated with both occult blood in stools (107) and neurologic symptoms (2).

TABLE 2. Plasmid genes associated with virulence in *S. flexneri*,[a] *S. sonnei*,[b] and *S. dysenteriae* 1

Gene	Relative position (kb)[c] and direction of transcript	Regulation	Protein product	Regulatory or effector function	References
Stb	0–6	Unknown	Unknown	Necessary for stable maintenance of 230-kb plasmid	88, 130
Rep	16–19	Unknown	Unknown	Necessary for replication of 230-kb plasmid	88, 130
virF	68←69	*virR* (?)[d]	30 kDa	Positive regulation of *virB* (*invE*, *ipaR*) and *virG* (*icsA*)	1, 69, 111, 112, 113, 124, 125, 149
invA (*mxiB*)	115←116	*virB* (*invE*, *ipaR*)[e]	38 kDa	Necessary for invasion (orients *ipa* gene products in outer membrane)	1, 61, 90, 123, 125, 149, 152
mxiA (may be homologous to *invK*)	118←120	*virB* (*invE*, *ipaR*)	76 kDa	Same as above[f]	61, 123, 149
invJ	122←124	*virB* (*invE*, *ipaR*)	Unknown	Same as above[f]	61, 123, 149
invH	125←127	Unknown	Unknown	Necessary for invasion (role unknown)[f]	123, 149, 150
invF	127←128	Unknown	Unknown	Same as above[f]	123, 149, 150
invG	128→129	*virB* (*invE*, *ipaR*)	24 kDa[g]	Not necessary for invasion[h]	122, 123, 149, 150
ippI	129→130	*virB* (*invE*, *ipaR*)	18 kDa	Same as above[h]	4, 5, 122, 144
ipaB	130→132	*virB* (*invE*, *ipaR*)	62 kDa	Necessary for invasion (may mediate endocytic uptake of shigellae)	1, 4, 5, 14, 15, 54, 61, 69, 81, 90, 98, 113, 122, 123, 144, 146, 150
ipaC	132→133	*virB* (*invE*, *ipaR*)	43 kDa	Same as above	1, 4, 5, 14, 15, 54, 61, 69, 81, 90, 98, 113, 122, 123, 144, 146, 150
ipaD	133→134	*virB* (*invE*, *ipaR*)	38 kDa	Same as above (may mediate adherence of shigellae to host cell membrane)	1, 4, 5, 14, 15, 54, 61, 69, 81, 90, 98, 113, 122, 123, 144, 146, 150
ipaA	134→136	Same as above	78 kDa	Not necessary for invasion or positive Serény test (role unknown)	1, 4, 5, 14, 15, 54, 61, 69, 81, 90, 98, 113, 122, 123, 143, 144, 146, 150
virB (*invE*, *ipaR*)	137→138	*virF*	33 kDa	Positive regulation of *ipaABCD* and *inv AKJHFG*	1, 15, 69, 90, 149, 150
virG (*icsA*)	160→166	*kcpA*[i] and *virF*	120 kDa[j]	Associated with intra- and intercellular bacterial spread	8, 35, 79, 87, 98, 105, 113, 125
ipaH	Unknown (present in multiple copies)	Unknown	60 kDa	Unknown (may inhibit coagulation)	14, 57, 145
crb	Unknown (linked to IS*1*, may be homologous to *virF*)	Unknown	24 kDa	Binding of Congo red	22, 23
LPS^{+}[k]	Unknown	Unknown	12.5-kb locus encoding proteins of 23, 39, 42, and 48 kDa	Necessary for expression of form I O side chains of *S. sonnei*	73, 118, 158a
rfp[l]	9-kb plasmid	Unknown	41 kDa	Adds galactose of *S. dysenteriae* 1 O side chain to LPS core	51, 153, 154

[a] Genes designated *vir*, *mxi*, *ipa*, *ipp*, *ics*, and *crb* have been identified in *S. flexneri*.
[b] Genes designated *inv* have been identified in *S. sonnei*.
[c] Gene position relative to *Sal*I restriction fragment O of the *S. flexneri* 2a pMYSH600 plasmid (Fig. 2).
[d] The *virR* locus is an analog of the *E. coli osmZ* (*bglY*, *cur*, *pilG*, *drdX*) locus that affects DNA supercoiling.
[e] *virB* (*invE*, *ipaR*) has been shown to positively regulate *ipaBC*, *invG*, *invJ*, and *invK*. *virF* has been shown to positively regulate *virB* (*invE*, *ipaR*), *ipaBC*, *invG*, *invJ*, *invK*, and *virG* (*icsA*). Therefore, it is proposed that *invA* (*mxiB*), *mxiA* (*invK*), *invJ*, *invG*, and *ipaBCDA* are regulated by *virF* through the *virB* (*invE*, *ipaR*) intermediary. *invH* and *invF* are not regulated by *virF* or *virR* (*invE*, *ipaR*) (150).
[f] Insertions in this locus may have negative polar effects on expression of the *invA* gene.
[g] In *S. flexneri* 2a, an unnamed ORF that apparently corresponds to the *invG* locus of *S. sonnei* encodes a 24-kDa protein. Also, 14 kDa of an analogous ORF in *S. flexneri* 5 has been sequenced (144).
[h] Insertions in this locus may have negative polar effects on expression of the *ipaABCD* locus.
[i] Chromosomal gene linked to *purE* in *S. flexneri* and in *E. coli*.
[j] The *virG* gene product has been reported to be a 120-, 130-, or 140-kDa protein (based on relative mobility in SDS-PAGE). The *virG* ORF indicates a transcript size of 117 kDa.
[k] Partially mapped locus on the 180-kb *S. sonnei* virulence plasmid.
[l] The *rfp* locus is located on a 9-kb plasmid only in *S. dysenteriae* 1.

Genes Regulating Expression of the *Shigella* Virulence Plasmid

The discovery that many virulence determinants of *Shigella* species and EIEC strains are encoded by 180- to 230-kb plasmids has lead to a reevaluation of the role of some chromosomal genes in virulence. For example, loci linked to *purE* (105), *malA* (38), and *galU* (91) appear to regulate expression of the virulence plasmid, and these three loci are present in both *E. coli* and *Shigella* species.

Plasmid expression linked to *malA* (*ompR-envZ*). A Serény-negative spontaneous colony variant of *S. flexneri* 5 (M90TX) was restored to virulence after conjugal transfer of the *malA* gene of *E. coli* K-12 (38). Recently it has been shown that the *malA*-linked *ompR-envZ* locus also restores the Serény-positive phenotype to M90TX (7, 7a). The *envZ* gene product is a transmembrane osmolarity sensor that phosphorylates the product of the *ompR* gene, and the latter protein modulates transcription of porin proteins *ompF* and *ompC* (21). *S. flexneri* mutants with Tn*10* insertions in *envZ* or deletions in *ompR* are about 75% less invasive than the parent strain in HeLa cell monolayers. Since the residual invasive organisms are incapable of plaque formation in HeLa cell monolayers, a possible defect in intracellular multiplication or intercellular spread may also be present in *ompR-envZ* mutants. Expression of a *vir*::*lac* operon fusion in an unidentified plasmid virulence gene is enhanced by high osmolarity (300 mOsm), and modulation is not observed in an isogenic *ompR* deletion mutant (7). Thus, it could be speculated that products of the two-component *ompR-envZ* regulatory locus modulate the expression of the plasmid-encoded invasive phenotype when shigellae are exposed to the hypertonic colonic contents of the primate intestine.

Keratoconjunctivitis provocation (*kcpA*). The marker designated KcpA was originally identified in *S. flexneri* 2a hybrids that had lost the ability to evoke keratoconjunctivitis after the *purE* locus (12 min) was transferred from *E. coli* K-12 by conjugation or transduction (35). In the corneal epithelium, *kcpA* mutants invade individual epithelial cells, but the infection remains localized and keratoconjunctivitis (a positive Serény test) does not develop (158). The *kcpA* locus is associated with expression of a 120-kDa protein that is encoded by the *virG* (*icsA*) gene on the 230-kb virulence plasmid (79, 105). *S. flexneri* strains that are defective in either *kcpA* or *virG* (*icsA*) expression are fully invasive in the tissue culture model, but they multiply within localized areas of the cytoplasm of infected cells and do not spread to contiguous cells (87, 105, 158).

It has been suggested that *kcpA* mutants remain localized in the host cell cytoplasm because they fail to escape from endocytic vacuoles (158). However, electron micrographs of cells infected with nonspreading *virG* (*icsA*) mutants of *S. flexneri* indicate that these organisms can escape endocytic vacuoles (8), and nonspreading *E. coli* K-12 hybrids carrying the 230-kb plasmid of *S. flexneri* also lyse endocytic vacuoles (105). The latter hybrids are usually incapable of the intercellular spread and plaque formation in a tissue culture monolayer, but stable plaque-forming variants of *E. coli* K-12 that express the 120-kDa *virG* (*icsA*) protein product can be isolated at a low frequency. The plaque-forming phenotype and *virG* (*icsA*) expression are linked to the *purE* locus in these variant *E. coli-S. flexneri* hybrids (105). Therefore, an allele of the *Shigella kcpA* locus is apparently present, but rarely expressed, in the *E. coli* chromosome.

By selecting for the ability of an *S. flexneri* chromosomal gene library to restore the plaque-forming phenotype to an *S. flexneri* strain that has acquired the *E. coli* K-12 *purE* region, the putative *kcpA* gene has been cloned and sequenced (158). A 12-kDa protein product corresponding to the cloned ORF was identified in minicells. However, it has recently been noted that the sequence of the cloned *kcpA* gene is essentially identical to the carboxy-terminal sequence of the histonelike H1 protein product of the *osmZ* locus (62). The latter locus has been mapped at 27.5 min on the *E. coli* and *S. flexneri* chromosome. Hybridization studies show that there is a single *osmZ* coding sequence in the chromosome of *E. coli* and *S. flexneri* (27, 59). Thus, it appears that a truncated version of *osmZ* rather than *kcpA* was cloned, and the product of this multicopy clone may have spuriously transcomplemented the *kcpA* regulatory lesion (62).

Plasmid expression linked to *galU* (*virR*, *osmZ*). The chromosomal genes linked to *purE* (*kcpA*) and *malA* (*ompR-envZ*) are apparently necessary for the positive regulation of key plasmid virulence determinants. However, a chromosomal locus designated *virR* is necessary for repression of these determinants when shigellae are grown at 30°C (91). Since transposon insertions in *virR* allow constitutive expression of the invasive phenotype at 30 and at 37°C, this locus is not strictly a virulence gene. The *virR* mutants are fully virulent in animal models. Nonetheless, the *virR* regulatory loop may mediate the bacterial response to environmental conditions outside the primate host by inhibiting the expression of virulence determinants and conserving energy (89).

The *virR* locus is linked to *galU* (27 min) in the *S. flexneri* chromosome (91), and an analogous locus is present in *E. coli* since the transduced *galU* gene from *E. coli* K-12 can reconstitute temperature regulation of the invasive phenotype in an *S. flexneri* strain that has suffered a deletion in *virR* (60). The *galU* gene is also linked to the *osmZ* locus, which controls DNA supercoiling in *E. coli*, and similar phenotypic alterations are induced by an *osmZ* mutation and by a *virR*::Tn*10* mutation in an *E. coli* background. In addition, Southern hybridization indicates that the *virR*::Tn*10* gene integrates into the *E. coli* chromosome at the *osmZ* site. Thus, *virR* mutants are allelic with a growing list of similar mutants including *osmZ*, *hns*, *bglY*, *cur*, *pilG*, and *drdX* (27, 62). The product of this gene (protein H1) is a histonelike protein, and the binding of H1 may change local topological microdomains of target DNA.

Mutations in *drdX* negatively regulate two divergently oriented *pap* operon promotors, suggesting independent interaction between a number of regulatory genes and the H1 gene product (44). By analogy, *virR* could mediate plasmid gene expression through changes in DNA supercoiling. This hypothesis is supported by the observation that small plasmids in *S. flexneri virR*::Tn*10* mutants are relaxed when grown at 30°C but supercoiled in the *virR*$^+$ parent. In addition, coumermycin A_1, a gyrase inhibitor that relaxes DNA, releases the wild-type *S. flexneri* from temperature-mediated repression of the invasive phenotype (27). Thus it would appear that *virR* represses the invasive phenotype in *S. flexneri* grown at 30°C by inducing supercoiling in invasion plasmid DNA.

Plasmid expression linked to *glpK*. An opaque colony variant of *S. flexneri* 2a designated 2457O was originally used to document the avirulence of noninvasive *Shigella* strains (76). This strain was subsequently found to be deficient in glycerol kinase activity, and selection for transfer of the *glpK* gene in conjugation or transduction experiments with the virulent 2457T parent strain restored the Serény-positive phenotype in approximately half of the recipients (71).

However, the linkage of virulence to *glpK* is weak, and the 2457O variant is not restored to the invasive phenotype by transduction of the *glpK* locus of *E. coli* K-12 (71). Subsequent work has shown that virulence plasmid loci are not expressed in the 2457O variant (54), and recent observations suggest that the opaque colony morphology reflects spontaneous insertions in plasmid DNA rather than in a chromosomal locus (91a).

PLASMID GENES ASSOCIATED WITH VIRULENCE

Large plasmids were first detected about 12 years ago in *S. flexneri* 2a (72), and the essential role of plasmids in virulence was established shortly thereafter in both *S. sonnei* (118) and *S. flexneri* (119). Subsequently it was shown that virulence plasmids are also present in other serotypes of *S. flexneri*, *S. dysenteriae*, *S. boydii*, and EIEC (55, 115, 117). Endonuclease digestion and Southern hybridization indicate that the virulence plasmids of *Shigella* species and EIEC are essentially homologous but restriction sites vary with the species and serotype (55, 115). The ability to invade tissue culture cells is transferred to *E. coli* K-12 by the conjugal mobilization of 220- to 240-kb plasmids from *S. flexneri* 5 (116). The invasive phenotype can also be transferred by a 180-kb plasmid from *S. sonnei* (151). The characteristics conferred by these invasion plasmids can be illustrated in vitro with *S. flexneri* 5 minicells that carry only plasmid DNA (55) (Fig. 1). Plasmid-encoded proteins mediate the attachment and endocytic uptake of minicells by HeLa cells (Fig. 1A). Following ingestion, the endocytic vacuoles dissipate in a plasmid-dependent process and the minicells are released into the cytoplasm (Fig. 1B).

A 230-kb plasmid of *S. flexneri* 2a has been subjected to *Sal*I endonuclease digestion, and 23 fragments have been identified and mapped (124, 125) (Fig. 2). In the context of this *Sal*I map, genetic data from the *S. flexneri* 2a, *S. flexneri* 5, and *S. sonnei* plasmids are summarized in Table 2. The relative position and direction of transcription of virulence-associated loci have been deduced from the literature, and the loci are listed in a clockwise order starting with the Stb marker that is located in *Sal*I fragment O in *S. flexneri* 2a (88, 125).

Stb and Rep (Stable Maintenance and Replication)

Analysis of plasmid incompatibility has suggested that *S. flexneri* serotypes 1 to 5 and *S. sonnei* belong to the IncFI group, whereas *S. flexneri* serotype 6 and *S. boydii*, *S. dysenteriae*, and EIEC are compatible with IncFI plasmids (88). The Rep region of *Sal*I fragment C from the *S. flexneri* 2a virulence plasmid shares homology with the RepFIIA family of replicons, and this region is responsible for incompatibility with IncFI and IncH1 plasmids (88). Presumably, the Rep region is also responsible for homology seen with a RepFIC probe in clinical isolates of EIEC and *Shigella* species (130). Along with a contiguous region on *Sal*I fragment O (Stb, stable maintenance), the Rep locus is necessary for replication and maintenance of virulence plasmids.

virF (Positive Regulator of the Plasmid Virulence Regulon)

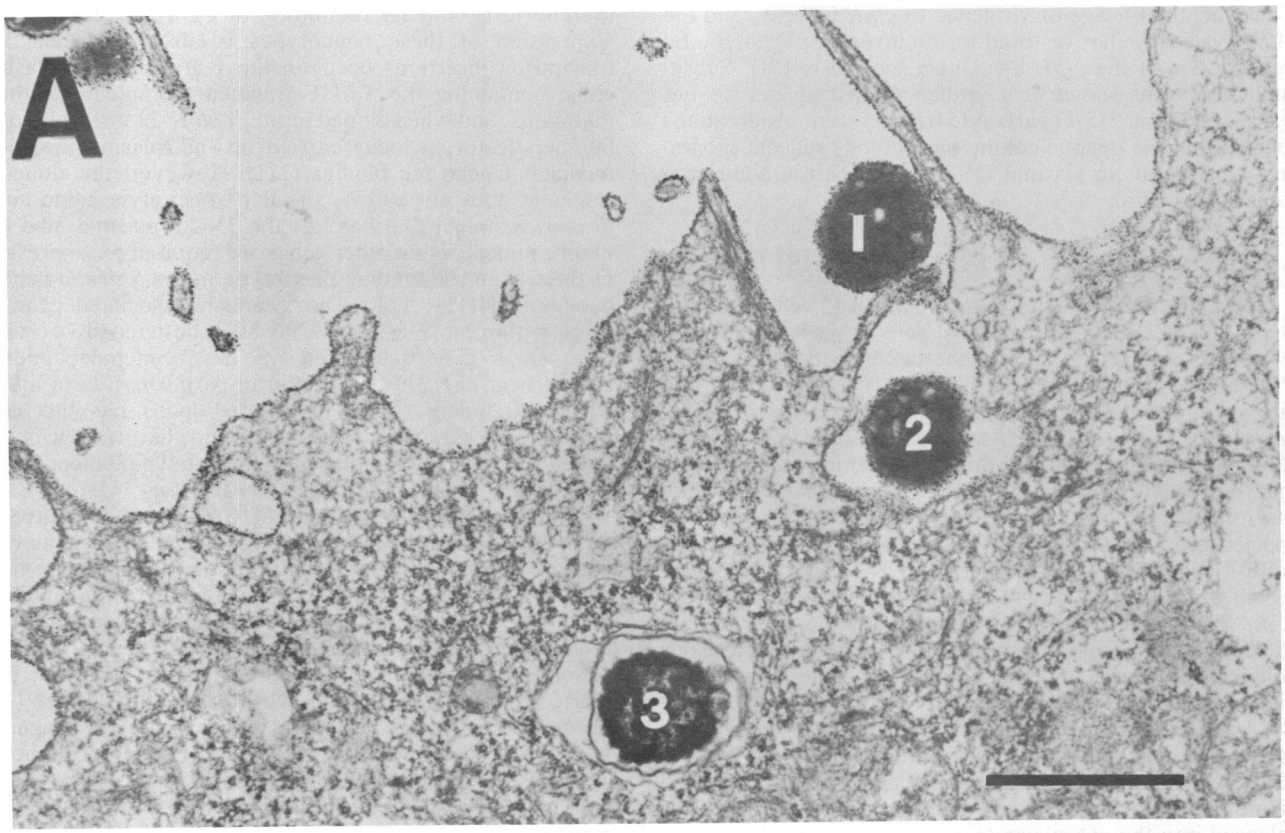
Located approximately 50 kb from the origin of replication, the *virF* locus was first identified by a spontaneous deletion in *Sal*I fragment F that resulted in the simultaneous loss of four virulence-associated phenotypes: Pcr (Congo red binding), Inv (invasion of tissue culture cells in vitro), Ser (Serény test), and Igr (inhibition of growth) (124) (Fig. 2). Expression of these phenotypes is also lost when Tn*5* transposon insertions occur in the F fragment. A plasmid clone containing the *Sal*I F fragment complements these mutations, and when cloned into *E. coli* K-12 in a high-copy-number vector, a locus carried on this plasmid fragment mediates Congo red binding (111). However, the cloned F fragment does not convey the Pcr^+ phenotype upon an *S. flexneri* recipient that has lost the 230-kb plasmid, and this finding indicates that other genes are required for expression of the Congo red-binding phenotype in the *Shigella* genetic background (111, 124). The virulence-associated gene located within the F fragment has been designated *virF* (113) (Fig. 3). A gene designated *crb* has been independently cloned from the 240-kb plasmid of *S. flexneri* 2a in a 9-kb *Bam*HI fragment (22). This cloned locus has the same phenotypic characteristics as *virF*, and, like *virF*, it is also linked to IS*1* elements that may mediate spontaneous deletions in this region of the plasmid (124).

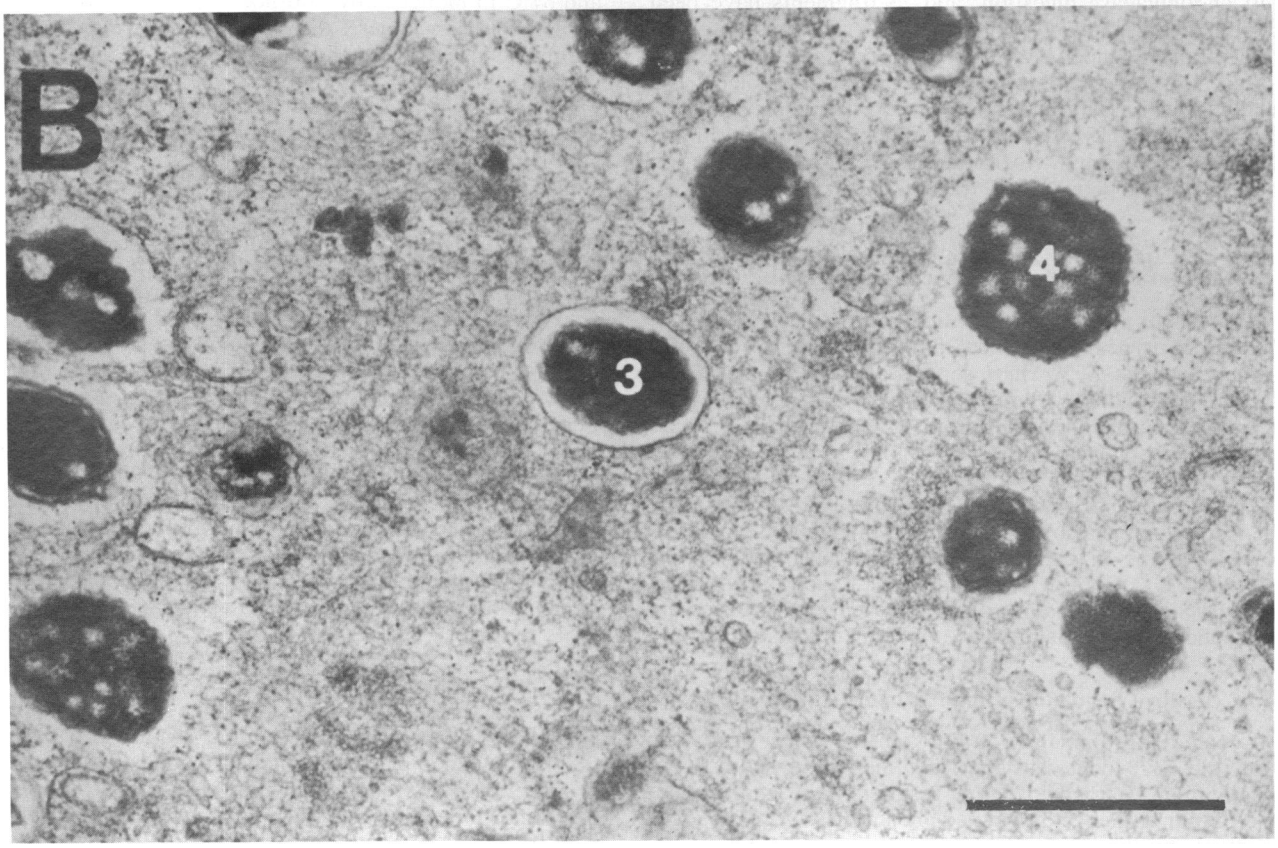
The functional gene product of *virF* is a 30-kDa protein, but a 24-amino-acid signal peptide-like sequence may be cleaved during passage through the inner membrane, yielding a 27-kDa protein that has been detected in minicells (112). The *virF* gene product plays a central role in positive regulation of the plasmid virulence regulon. It directly activates transcription of the *virG* gene (113), and it indirectly activates *ipaABCD* (69, 113, 149) and *invAKJHF* (149) plasmid loci through a second gene variously designated *virB*, *ipaR*, or *invE* (Fig. 3). This central regulatory function accounts for the concomitant loss of four virulence-associated phenotypes in the *virF* mutants (124, 125). The cloned *virF* locus is temperature regulated in both *S. flexneri* and *E. coli* K-12 backgrounds (111), but it is expressed at both 30 and 37°C in minicells that do not carry chromosomal regulatory loci such as *virR* (*osmZ*) (112). Therefore, the *virR* chromosomal gene may regulate plasmid-encoded invasion determinants at 30°C by inhibiting *virF* expression (1).

Since many *Shigella* genes can transfer the Pcr^+ marker to *E. coli* when cloned on high-copy-number vectors (16, 23, 67a, 111), the location of the structural genes encoding Congo red-binding protein(s) in *Shigella* species is unclear. Nonetheless, a 101-kDa protein (or protein complex) has recently been identified as a heme-binding outer membrane component that may also be the Congo red-binding protein (137). A 6.1-kb *Pst*I plasmid fragment which confers expression of the 101-kDa protein and the Pcr^+ phenotype in a plasmid-cured *S. flexneri* background does not reconstitute the invasive phenotype. Conversely, a 37-kb plasmid fragment that does reconstitute invasion does not confer the Pcr^+ phenotype (90). Even though the Inv^+ and Pcr^+ phenotypes are expressed independently, they are synergistically related in that pretreatment of shigellae with Congo red or hemin increases the invasion of HeLa cells (24). Since Pcr^+ *S. flexneri* strains are more hydrophobic than isogenic plasmid-cured strains (128), the Pcr^+ phenotype may indirectly reflect a characteristic of the bacterial surface that enhances the initial interaction of shigellae with host cells.

Plasmid Invasion Genes

The first successful attempt to clone the invasive phenotype of *S. flexneri* 5 into an isogenic strain that had lost the 230-kb virulence plasmid indicated that the genes encoding this phenotype were located within a 37-kb plasmid segment (90). The cloned invasion region corresponds to *Sal*I restriction fragments D, H, P, and B in the 230-kb plasmid of *S.*


A
1
2
3


B
3
4

flexneri 2a (123, 125) (Fig. 2 and 3). The *Sal*I site between the H and P fragments is apparently missing in the *S. flexneri* 5 virulence plasmid (90). This is an example of the microheterogeneity that characterizes the invasion plasmids of all *Shigella* species (55, 115). Analysis of Tn*5* insertion mutants indicates that the invasion region can be divided into five subgroupings of loci identified as regions 1 through 5 (5, 123) (Fig. 3). These regions include the genes designated *invA* through *virB* (Table 2).

***virB* (*invE*, *ipaR*).** A regulatory locus necessary for expression of the invasive phenotype is located within *Sal*I fragment B of the *S. flexneri* 2a virulence plasmid, and the cloned gene has been designated *virB* (1). Complementation of noninvasive insertion mutants within *virB* (Fig. 3, region 1) indicates that expression of the "invasion plasmid antigens" of the *ipa* locus (region 2) is positively regulated by *virB* (1). Analogous genes have been designated *ipaR* in *S. flexneri* 5 (15) (Fig. 4) and *invE* in *S. sonnei* (150) (Table 2). Expression of *lac* fusions in a region 2 gene (*ipaB*) and in a region 3 gene (*invJ*) indicates that the *invE* gene product positively regulates genes in these regions in a dose-dependent manner (150). In addition, the *ipaR* analog of *virB* and *invE* positively regulates expression of the Pcr^+ marker that is encoded by loci outside the plasmid invasion region (15). These results suggest that the *virB* (*ipaR*, *invE*) gene mediates in *trans* the control of an extensive regulon of virulence-associated loci in the *Shigella* virulence plasmid. Northern blot analysis shows that transcription of *virB* is positively regulated by *virF* (1). Tn*3-lac* fusions in the *S. sonnei invE* gene indicate that the transcription of this gene is also positively regulated by *virF* (150).

The mobility of the protein product of *virB* in sodium dodecyl sulfate (SDS)-polyacrylamide gels indicates a molecular mass of 33 kDa (1), whereas the *ipaR* protein has been estimated to be 34 kDa (15) and the *invE* protein has been estimated at 35 kDa (150). Nonetheless, the nucleotide sequence of these loci is essentially identical, and the ORF encodes a hydrophilic protein product with a pI of 9.7 (15). The *virB* (*ipaR*, *invE*) sequence exhibits striking homology with the ORFs of both the ParB protein of bacteriophage P1 (15, 150) and the SopB protein of F plasmids (150). Since the latter proteins bind to palindromic DNA sequences such as those located at the end of *ipaB* and *ipaC* ORFs in region 2, the *virB* (*ipaR*, *invE*) protein product may regulate *ipa* expression by binding to these domains.

***ipaABCD*, *ippI*, and *invGF*.** Immunoblots with serum from monkeys or humans infected with *Shigella* species demonstrate a consistent serum immune response that recognizes five plasmid-encoded proteins (54, 69, 90, 98, 122, 150). The largest of these proteins is the product of the *virG* (*icsA*) locus, which is located outside the invasion region. The other proteins are encoded by the *ipa* locus, which corresponds to invasion region 2 in the *S. flexneri* 2a virulence plasmid (Fig. 3) (123). Since the latter proteins were originally designated *a* (78 kDa), *b* (62 kDa), *c* (43 kDa), and *d* (38 kDa) in order of descending molecular mass (54), the corresponding genes have been named *ipaABCD* (14) (Fig. 4). Antibody evoked by infections with *S. flexneri* 2a cross-reacts with proteins of similar molecular mass in *S. flexneri* serotypes 1 to 6, *S. dysenteriae*, *S. boydii*, *S. sonnei*, and EIEC (54, 92, 117). The cloned *ipaBCD* region can also be used as a specific DNA probe to identify enteroinvasive organisms (146). These observations suggest that the *ipa* locus is highly conserved in *Shigella* invasion plasmids, and the prominent immune response evoked by the quantitatively minor protein products of this locus attests to their unique association with enteroinvasive pathogens.

The nucleotide sequence of the *ipaBCD* region cloned from the *S. flexneri* 5 invasion plasmid reveals three ORFs corresponding to the predicted 62-, 42-, and 37-kDa proteins (4, 122, 144). In addition, an ORF encoding an 18-kDa protein (the *ippI* locus) is located immediately adjacent to the *ipaB* gene (4, 122, 144) (Fig. 4). Contiguous to the *ippI* gene in the invasion plasmid of *S. flexneri* 2a is an ORF encoding a 24-kDa translation product (122). Published restriction maps of this region suggest that this ORF probably corresponds to the *invG* gene recently identified in *S. sonnei* (150). A Tn*3-lacZ* gene fusion in *invG* indicates that this gene is transcribed in the same direction as the *ipa* locus. An additional gene (*invF*), identified by an upstream Tn*3-lacZ* fusion, is also transcribed in the same direction as the *ipa* locus (149, 150). The *invF* gene probably marks the end of invasion region 2 (123) (Fig. 3). Putative promoter sequences have been detected upstream of 24-kDa (*invG*) and 18-kDa (*ippI*), *ipaC*, *ipaD*, and *ipaA* ORFs (4, 122). S1 nuclease protection indicates that an RNA transcript originates upstream of the locus encoding the 24-kDa protein of *S. flexneri* 2a and that this transcript may traverse the entire *ipa* locus (122). Northern blots indicate that transcripts including combinations of 24 kDa (*invG*), 18 kDa (*ippI*), *ipaB*, and/or *ipaC* are present in *S. flexneri* 5 (144) and *S. flexneri* 2a (122). In contrast, the downstream *ipaD* and *ipaA* genes are usually transcribed independently (122, 144).

Tn*5* or Tn*3-lacZ* insertions in *invF*, *invG*, *ippI*, *ipaB*, *ipaC*, and *ipaD* result in the loss of the invasive phenotype, whereas mutants with insertion mutations mapping within *ipaA* retain this phenotype (5, 122, 123, 150). Complementation experiments show that clones expressing the *ipaB* and *ipaC* protein products but not the 24-kDa (*invG*) and 18-kDa (*ippI*) products restore the invasive phenotype in mutants that have suffered Tn*5* insertions in *invG*, *ippI*, *ipaB*, or *ipaC*. Conversely, clones expressing the *invG* and *ippI* gene products do not restore the invasive phenotype to *ipaBC* insertion mutants (122). These data indicate that *ipaB* and *ipaC* are essential for expression of the invasive phenotype whereas *invG* and *ippI* are nonessential. Thus, insertion mutations in the latter genes, and also within the *invF* gene, may exert biological effects indirectly.

Hydropathy profiles of the deduced amino acid sequences of IpaBC are suggestive of hydrophilic proteins with no signal peptide sequence but with centrally located hydrophobic domains of 120 amino acids (IpaB) or 60 amino acids (IpaC) (4, 144). MAbs recognizing two epitopes in the amino-terminal end of IpaB bind to whole bacterial cells in

FIG. 1. Electron micrograph illustrating four stages in the invasion of HeLa cells by minicells isolated from *S. flexneri* serotype 5 strain M90TminII (55). The minicells were centrifuged onto the HeLa cells and incubated at 37°C for 30 min. The monolayers were then fixed in glutaraldehyde and osmium tetroxide, stained with colloidal thorium, and prepared for sectioning. The thorium provides an electron-dense marker surrounding external bacteria and the exterior plasma membrane so that the attachment (no. 1) and endocytosis (no. 2) of minicells can be differentiated from organisms taken up in endocytic vacuoles (no. 3). Within 30 min of internalization, the endocytic membrane dissipates into a vacuolar area surrounding the minicells (no. 4), and the organisms are subsequently found free in the HeLa cell cytoplasm. Bar, 1.0 μm. Reprinted from reference 55 with permission.

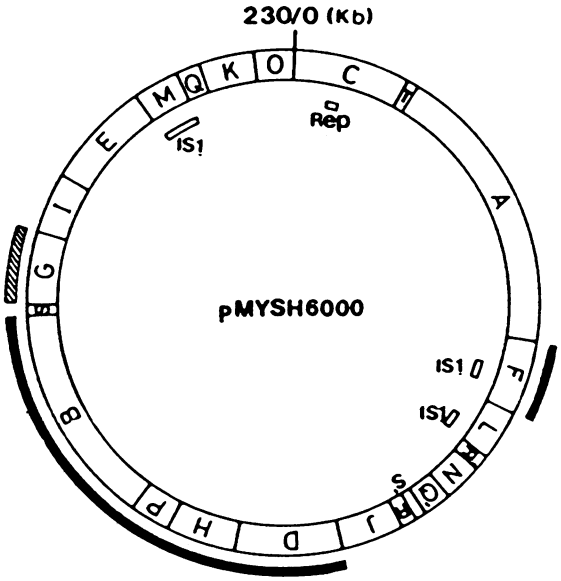


FIG. 2. Circular *Sal*I restriction map of *S. flexneri* 2a plasmid pMYSH6000. The area indicted by solid segments contains genes responsible for Ser^+ (Serény test), Inv^+ (invasion of cultured mammalian cells), Pcr^+ (binding of Congo red), and Igr^+ (inhibition of growth). Symbols: ▨, segment necessary for Ser^+ but not for Inv^+, Pcr^+, and Igr^+; □, IS*1*-like elements and the origin of replication (Rep). Reprinted with permission from Sasakawa et al. (125).

an enzyme-linked immunosorbent assay (ELISA) (61, 92). One of these MAbs, designated 2F1, partially inhibits the formation of plaques in baby hamster kidney (BHK) cell monolayers (92), indicating that the amino-terminal domain of IpaB is necessary for bacterial invasion or for intercellular bacterial spread. In contrast, three MAbs recognizing epitopes closer to the central hydrophobic domain of IpaB are much less reactive in the whole-cell ELISA (92), suggesting that this is a transmembrane protein region. These weakly reactive MAbs have no effect upon plaque formation in infected BHK monolayers. Three MAbs recognizing epitopes near the amino-terminal end of IpaC are strongly reactive in the whole-cell ELISA, but these reagents have no effect upon plaque formation. One MAb, designated 2G2, recognizes an epitope on the carboxy-terminal side of the central hydrophobic domain of IpaC, and pretreatment of *S. flexneri* with this MAb enhances plaque formation (92). This positive effect suggests that the carboxy-terminal portion of IpaC affects the expression of the invasive phenotype, and it should be noted that the hydropathy profile of the carboxy-terminal half of IpaC mirrors the hydropathy profile of the amino-terminal half of IpaB.

Results of immunofluorescence studies with polyclonal rabbit antiserum recognizing IpaC indicate that this protein is detectable on the bacterial surface only if shigellae are grown in the presence of Congo red (114), and pretreatment of bacteria with this dye also enhances expression of the invasive phenotype (24). In addition, shigellae that have invaded tissue culture cells are recognized by anti-IpaC antiserum (114). These data suggest that the transport and/or synthesis of Ipa proteins is enhanced by the binding of hydrophobic dyes and by growth within the host cell cytoplasm. However, a recent report indicates that growth of *S. flexneri* within tissue culture cells actually inhibits de novo expression of Ipa proteins (58). The latter experiments involved immunoprecipitation of radiolabeled *Shigella* proteins from monolayers that had been solubilized by relatively gentle detergent treatment, and interpretation of the results was complicated by the precipitation of many radiolabeled proteins in addition to the Ipa proteins. Nonetheless, it is possible that IpaC protein is transported to the outer membrane of *S. flexneri* growing in the host cell cytoplasm at the same time that de novo synthesis of Ipa proteins is repressed. Additional studies are required to fully characterize the regulatory patterns of Ipa protein expression during bacterial invasion and intracellular growth.

The hydropathy profile of the deduced IpaD amino acid sequence reveals the periodicity of a helical molecule with no extensive hydrophobic domains suggestive of a signal sequence or a transmembrane region. A critical role for IpaD in the invasion process can be inferred from observations obtained with noninvasive Tn*5* insertion mutants with mutations that map within the *ipaD* gene (5, 122). These mutants are less adherent to tissue culture cells than are wild-type shigellae; this may indicate that IpaD facilitates the initial interaction of shigellae with the surface of host cells (59a).

Although the molecular mobility of IpaA in SDS-polyacrylamide gel electrophoresis (PAGE) suggests a mass of 78 kDa (54), the recently sequenced *ipaA* ORF is indicative of a hydrophilic protein of only 70 kDa (143). Tn*5* insertions into the *ipaA* gene retain the invasive phenotype in the tissue culture model (5) and elicit a delayed-positive Serény reac-

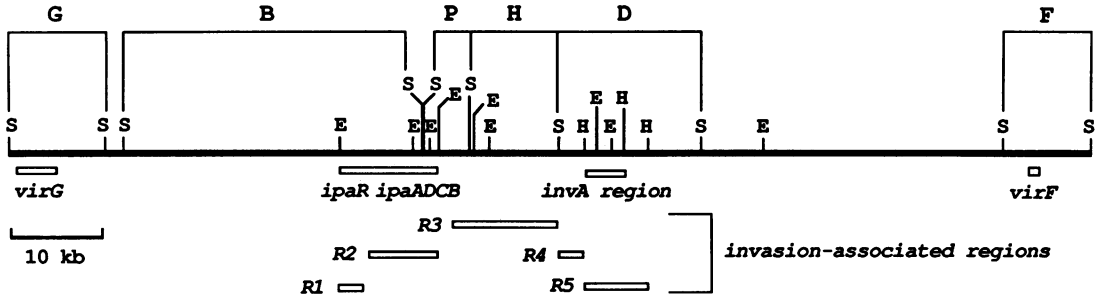


FIG. 3. Restriction map of *Sal*I fragments G, B, P, H, D, and F containing the known plasmid virulence genes of *S. flexneri*. The five invasion-associated regions were defined by Tn*5* insertions that eliminate expression of the invasive phenotype (123). The *virG* gene has also been designated *icsA*, and the *ipaR* gene has been designated *virB* or *invE*. Restriction sites: S, *Sal*I; E, *Eco*RI; H, *Hin*dIII. Figure courtesy of J. M. Buysse.

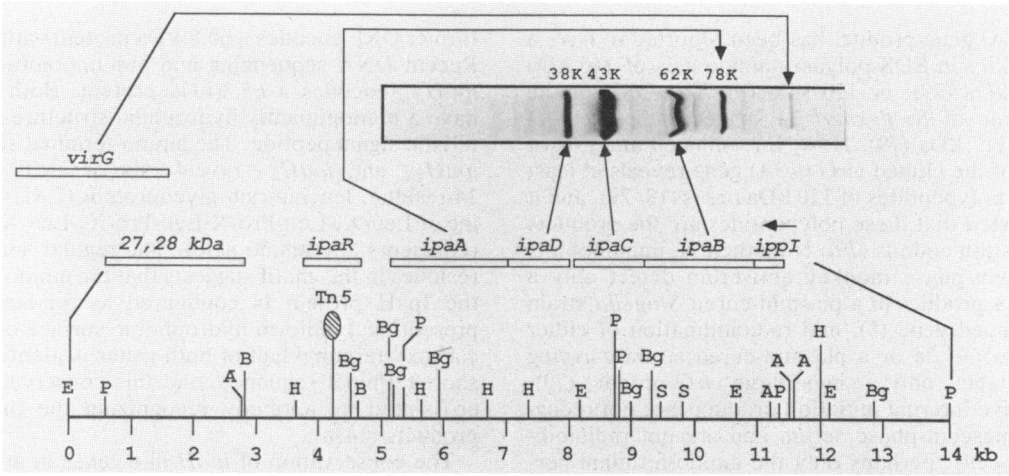


FIG. 4. Composite figure with an immunoblot showing the protein products of the invasion plasmid antigen (*ipa*) locus imposed above a restriction map of the encoding region of the *S. flexneri* 5 invasion plasmid. Also detectable in the immunoblot is the protein product of the *virG* (*icsA*) locus. The immunoblot was developed with a convalescent-phase serum sample from a rhesus monkey infected with *S. flexneri* 2a. The Tn*5* insertion mutant pHS1042 (90), which defines the *ipaR* (*virB*, *invE*) regulatory gene (15), is included in the mapped region. Restriction sites: E, *Eco*RI; P, *Puv*II; A, *Acc*I; B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II. Figure courtesy of J. M. Buysse.

tion. Therefore, the role of IpaA in virulence has not yet been ascertained.

***invAKJH* (*mxiAB*).** Tn*3-lac* fusion inserts within the *S. sonnei* invasion plasmid have defined four transcribed genes, designated *invAKJH*, that are necessary for expression of the invasive phenotype (149, 150) (Table 2). Restriction maps suggest that these genes correspond to invasion regions 3 and 5 of the *S. flexneri* 2a plasmid (123) (Fig. 3). Expression of *invAKJH* is under the positive control of the *virF-virB* (*invE-ipaR*) system (1, 61, 149, 150), but this locus differs from the genes of invasion regions 1 and 2 in the direction of transcription (Table 2) (61, 149, 150). Published restriction maps suggest that *invA* should map within invasion region 5 and *invKJ* should map within invasion region 3 on the *S. flexneri* 2a plasmid. Restriction analysis also indicates that an *S. sonnei* gene designated *invH* should map at the junction of invasion region 3 and region 2 in the *S. flexneri* 2a virulence plasmid (123, 149, 150). Since results of precise mapping and sequencing of invasion regions 3, 4, and 5 have yet to be published, the molecular mass of *invKJH* gene products is unknown. However, *invA* insertion mutants are complemented by a cloned fragment from the *S. sonnei* plasmid which expresses a 38-kDa protein (152).

Deletion mutants eliminating invasion region 3, 4, or 5 are noninvasive even though they express the gene products of invasion regions 1 and 2 (15). A noninvasive, *inv*::*lacZ S. flexneri* 2a fusion mutant designated BS226 has been found to bind 80% less 2F1 MAb (anti-IpaB) and 66% less 2G2 MAb (anti-IpaC) in a whole-cell ELISA (61). Restriction maps indicate that the BS226 fusion is homologous to *invA* mutants. Similar inhibition of MAb binding was observed in a whole-cell ELISA with *inv*::*lacZ* fusion mutants BS232 and BS230 that map within an 8-kb *Eco*RI-*Sal*I fragment of invasion region 3 (61). It is possible that the genes defined by the latter fusions are homologous to *invKJ*. Quantitative immunoblots of isolated membrane fractions have recently shown that neither IpaB nor IpaC is transported to the outer membrane in BS226 or in another *inv*::*lacZ* fusion mutant (BS260) that is probably analogous to BS232. The invasion plasmid antigens accumulate within the inner membrane in these mutants (1a). Therefore, the *S. flexneri* 2a gene defined by the *inv*::*lacZ* insertion BS226 has been designated *mxiB* and the gene defined by the BS260 or BS232 fusions has been designated *mxiA* (*mxi*, membrane expression of Ipa) (61) (Table 2). Rabbit antiserum raised against the BS260 fusion protein recognizes a 76-kDa protein in immunoblots of an *S. flexneri* 5 whole-cell lysate (1a).

virG (*icsA*) (Plasmid Gene Associated with Intercellular Bacterial Spread)

The *virG* locus was first identified by transposon insertions within *Sal*I fragment G, located approximately 20 kb from invasion region 1 in the *S. flexneri* 2a plasmid (Fig. 2 and 3). *virG* insertion mutants are Inv^+, Pcr^+, and Igr^+, but Ser^- (125). As was explained in the description of the chromosomal *kcpA* locus, the Ser^- phenotype of *virG* mutants reflects an inability of intracellular organisms to spread within the cytoplasm of infected cells. Since this genetic defect precludes the infection of contiguous cells in tissue culture monolayers or within the corneal epithelium (79, 87, 105, 158), the homologous locus in *S. flexneri* 5 has also been designated *icsA* (intercellular spread) (8).

The *virG* (*icsA*) gene product was originally identified as the fifth invasion plasmid antigen of *S. flexneri* 5 (98), and extrinsic radioiodination of whole cells has shown that this protein is exposed on the bacterial surface (79). A unique property of strains that express the *virG* protein is a polar deposition of F actin (filamentous actin) around organisms in the cytoplasm of infected HeLa cells. This F actin sometimes gives the appearance of an elongated tail, and these actin tails are often found associated with bacteria in protrusions of the host cell plasma membrane (8). These deformations may represent the first stage in intercellular bacterial spread. Since the intracellular movement of organisms expressing *virG* (*icsA*) is reversibly inhibited by cytochalasin D (8, 105), the deposition of F actin apparently provides a motive force. The deduced amino acid sequence of *virG* is not indicative of a signal sequences or hydrophobic regions that might be associated with the outer membrane (79), but a remarkable feature of the sequence is a series of repeating motifs in the amino-terminal one-third of the molecule.

The *virG* (*icsA*) gene product has been reported to have a molecular mobility in SDS-polyacrylamide gels of 140 kDa (98, 105), 130 kDa (79), or 120 kDa (8). The ORF of the cloned *virG* gene of *S. flexneri* 2a suggests a protein of approximately 117 kDa (79). However, minicell analysis of the product(s) of the cloned *virG* (*icsA*) gene reveals at least nine nonvector polypeptides of 130 kDa or less (8, 79), and it has been suggested that these polypeptides are the products of internal initiation codons (79). Nonetheless, immunoblots with convalescent-phase monkey antiserum detect only a single large *icsA* product in a plasmid-cured *Shigella* strain carrying the cloned gene (8), and radioiodination of either wild-type *S. flexneri* 2a or a plasmid-cured strain carrying only *virG* also labels only a single large *virG* product (79). Since the putative internal initiation products are not recognized by convalescent-phase serum and are not radioiodinated in whole cells, perhaps only the amino-terminal portion of the largest protein product is exposed on the bacterial surface. Alternatively, it has recently been suggested that multiple *virG* polypeptides seen in minicells are aberrant translation products of the *virG* mRNA in that they are translated only in the absence of the *kcpA* chromosomal gene product. Thus, the *kcpA* protein has been described as a posttranscriptional activator that favors the transcription of a functional 120-kDa *virG* product (160).

ipaH (Multicopy Invasion Plasmid Antigen Gene)

When the *ipaBCD* genes were cloned from *S. flexneri* 5 into a λgt11 expression vector, the cloned genes were identified by colony lifts and immunoblots with antibody from rabbits that had been immunized with crude fractions of plasmid-encoded proteins (14). A subset of clones that expressed a 60-kDa protein was recognized by this rabbit serum, but affinity-purified antibody reacting with this protein did not cross-react with the 62-kDa IpaB protein. In addition, these clones did not hybridize with λgt11::*ipaB* (14, 15a, 57). Since a new invasion plasmid antigen had apparently been cloned in these experiments, the gene was designated *ipaH* (57).

The *ipaH* gene is unique in that five complete or partial copies are present on the invasion plasmids of the various *S. flexneri* serotypes and multiple copies are also found on the invasion plasmids of other *Shigella* species and EIEC (57). In addition, multiple copies of *ipaH* are present on the *Shigella* and EIEC chromosome but not on the chromosome of nonpathogenic *E. coli* strains (145). The copies of *ipaH* have been characterized by Southern hybridization of *Hin*dIII digests of *S. flexneri* 5 plasmid DNA with a λgt11::*ipaH* probe. These genes have been designated $ipaH_{9.8}$, $ipaH_{7.8}$, $ipaH_{4.5}$, $ipaH_{2.5}$, and $ipaH_{1.4}$ on the basis of the size of the hybridizing DNA fragment (57). $ipaH_{7.8}$ and $ipaH_{4.5}$ have been mapped to *Sal*I fragment B between *virG* and *ipaR* (within 10 kb of the latter gene). Northern blot analysis indicates that both $ipaH_{7.8}$ and $ipaH_{4.5}$ are transcribed in vitro whereas $ipaH_{2.5}$ and $ipaH_{1.4}$ are unexpressed, truncated sequences (143a). It is clear that one or more copies of *ipaH* is expressed during *Shigella* infections because specific antibody has been detected in convalescent-phase human serum (99a). Cloning of $ipaH_{7.8}$ in an *E. coli* background does not confer Congo red binding or the invasive phenotype, and, unlike other plasmid virulence genes, expression of *ipaH* is independent of temperature regulation or induction by *virF* (57).

The DNA sequence of the complete $ipaH_{7.8}$ gene and the amino-terminal half of $ipaH_{4.5}$ has been published, and the former ORF encodes a 60.8-kDa protein with a pI of 5.9 (57). Recent DNA sequencing and immunoblotting indicate that $ipaH_{4.5}$ encodes a 65.3-kDa protein. Both IpaH products have a predominantly hydrophilic structure with no characteristic signal peptide. The amino-terminal sequence of both $ipaH_{7.8}$ and $ipaH_{4.5}$ reveals six or eight evenly spaced, 14-residue, leucine-rich glycoprotein (LRG) motifs consisting of Leu-X_2-Leu-Pro-X-Leu-Pro-X_2-Leu-X_2-Leu (where X represents any amino acid). The regular spacing of leucine residues in this motif suggests that the amino-terminal half of the IpaH protein is configured as an amphipathic helix presenting a uniform hydrophobic surface on one side. The carboxy-terminal half of both genes is identical except for a short terminal sequence, and this conserved portion elicits cross-reacting antibody recognizing the other *ipaH* gene products (143a).

The conservation of *ipaH*-like genes in at least two complete plasmid copies suggests a powerful selection for the expression of these proteins. A possible explanation of the role of these proteins in virulence involves the amino-terminal, LRG-like motifs. These leucine-rich repeats are similar to the LRG-like motifs of the platelet glycoprotein GPIbα that binds von Willebrand factor, triggering conversion of prothrombin to thrombin in the process of fibrin clot formation and in the initiation of inflammatory processes. The only other example of LRG-like motifs in a prokaryotic protein is found in the YopM protein of *Yersinia pestis* (82). Culture supernatants of *Y. pestis* containing YopM inhibit coagulation, and *yopM* mutants of *Y. pestis* exhibit greatly decreased virulence in mice (81). Therefore, it has been suggested that YopM competitively inhibits the normal interaction of von Willebrand factor and/or thrombin with platelets. This competitive effect should prevent platelet adhesion to the exposed subendothelium of injured vessels and inhibit normal thrombus formation and inflammation.

The similarity of the LRG-like motifs of YopM and IpaH (50% identity in a 93-amino-acid overlap) indicates that IpaH may also act to modify the host response to *Shigella* infection. For example, the IpaH protein could inhibit both thrombus formation and the recruitment of inflammatory cells into the lamina propria during the early stages of infection. Thus, dissemination of shigellae within the mucosa could be facilitated, and inhibition of thrombin-induced coagulation could also exacerbate the loss of blood into the stools of dysenteric patients. Final characterization of the role of IpaH in virulence awaits the construction of $IpaH^-$ mutants, and the recent sequencing of $ipaH_{7.8}$ and $ipaH_{4.5}$ should allow the elimination of these plasmid genes by site-directed mutagenesis through gene replacement.

DISCUSSION OF VIRULENCE IN *SHIGELLA* SPECIES

Genetic Basis of Virulence in Enteroinvasive Pathogens

With the possible exceptions of the *flu* (fluid accumulation) locus (32, 116, 141, 159) and the chromosomal copies of *ipaH* (145), there are presently no identified chromosomal virulence-associated loci in *Shigella* species that do not have counterparts in the genome of *E. coli*. In the latter species, for example, the *iucABCD* and *iutA* genes of the aerobactin system are present in the *E. coli* pColV plasmid as a transposonlike element flanked by two inverted IS*1* repeat elements, and this configuration has been shown to promote cointegrate formation and transposition of the entire aerobactin system to other replicons (25). Flanking regions of the chromosomal aerobactin locus in *S. flexneri* also include

both a copy of IS*1* and a gene encoding a colicin that competes with ColV for receptor binding (106). This genetic similarity suggests that a primordial *Shigella* species could have acquired aerobactin by the integration of genes from the *E. coli* pColV. Likewise, SLT-I of enterohemorrhagic *E. coli* is identical to the Shiga toxin of *S. dysenteriae* 1. The former toxin is encoded by a lysogenic *E. coli* phage, but phage-flanking sequences have not been detected in the *S. dysenteriae* 1 chromosome (97). Therefore, it is possible that enterohemorrhagic strains of *E. coli* have acquired the *stx* chromosomal locus from *S. dysenteriae* 1 through phage conversion.

Given the remarkable similarity of the *E. coli* and *Shigella* genomes, it is apparent that the key event in evolution of the enteroinvasive phenotype was the acquisition of heterologous genes that are now carried on 180- to 230-kb virulence plasmids. The inference that plasmid virulence genes were acquired from another genus is based on the nucleotide sequences of *virF*, *virB*, and *ipaBC*, which are unusually rich in A and T; i.e., the A+T content of these plasmid genes varies from 70 to 73%, compared with an average of 50% in *E. coli* and *Shigella* chromosomal genes (1, 4, 112).

The metabolic burden imposed by the expression of the plasmid invasion regulon is implicit in the slow growth of invasive *Shigella* strains compared with noninvasive strains (124) (Igr^+, Fig. 2). Therefore, an intricate system of plasmid regulation has evolved from chromosomal genes that are present in noninvasive strains of *E. coli* as well as *Shigella* species. For example, *kcpA*, *ompR-envZ*, and *virR* (*osmZ*) are present on the chromosome of *E. coli* strains that do not carry a virulence plasmid. Repression of the plasmid invasion regulon by *ompR-envZ* and *virR* occurs under the low-temperature and hypotonic conditions routinely encountered by shigellae that have been shed from the intestinal tract. Conversely, ingested shigellae encounter an increasing gradient of osmolarity at mammalian body temperature during transit of the intestinal tract, and expression of the enteroinvasive phenotype in response to these environmental conditions offers shigellae the opportunity to escape the fully occupied environmental niche of the bowel lumen and to become the predominant species in the extreme environment of the enterocyte cytoplasm (93).

Biochemical Basis of Virulence in Enteroinvasive Pathogens

Impressive progress has been made during the last 10 years toward identification of the gene products of the *Shigella* invasion plasmid (Table 2), but this table also reveals the paucity of information concerning the functional interactions of these bacterial proteins with mammalian cells. Induction of invasion regulon expression is the most likely raison d'être for *virF* and *virB* (*ipaR*, *invE*), and the *invAKJ* (*mxi*) gene products are apparently necessary for transport and/or assembly of IpaB and IpaC proteins in the bacterial membrane. However, only *invA* insertion mutants have been restored to virulence by complementation with a functional *invA* gene (152), and the possibility remains that the insertion mutants that define the upstream *invKJ* loci inhibit expression of the invasive phenotype via polar effects on *invA*. Likewise, the *invHFG* gene products could be accessory proteins or structural components of the invasion determinant, but the noninvasive phenotype of *invHFG* insertion mutants could also reflect nonspecific polar effects on downstream expression of the *ipa* locus. The vigorous anti-Ipa antibody response induced by *Shigella* infection, the surface-exposed outer membrane location, and the modification of the invasive phenotype by MAbs recognizing *ipaBC* gene products make these proteins the most likely structural components of *Shigella* invasion determinants.

Plasmid-encoded invasion determinants mediate the endocytic uptake of shigellae by mammalian tissue culture cells in the sequential stages illustrated in Fig. 1. The bacteria first interact with the plasma membrane by forming areas of close apposition (<15 nm) that have the appearance of receptor-ligand interactions (47, 48, 53, 56). Under conditions that inhibit host cell endocytic activity, an *S. flexneri* strain that has lost the invasion plasmid adheres at only 1/10th the level of an isogenic invasive strain and both the adherent and the invasive phenotypes are eliminated by pretreatment of the latter strain with trypsin (104). This observation indicates that plasmid-encoded surface proteins are necessary for both adherence and invasion in *Shigella* species, and the poor adherence of *ipaD* insertion mutants suggests that the adherence stage could be mediated by the product of this gene.

The ingestion of attached shigellae is characterized by the accumulation of F actin in the cytoplasm underlying adherent bacteria (19). F-actin polymerization can be inhibited by cytochalasin B, and this fungal metabolite greatly reduces the uptake of shigellae (52). Soluble ligands are ingested by receptor-mediated endocytosis in coated pits surrounded by clathrin, and clathrin redistribution can be inhibited by depletion of intracellular potassium ions. Since potassium depletion also inhibits the uptake of shigellae (20), it could be postulated that the binding of plasmid-encoded bacterial ligands to host cell receptors mediates clathrin redistribution, leading to bacterial invasion. The second signal that mediates the polymerization of F-actin microfilaments under the attached bacterium has not been identified, but is clear that calcium ion fluxes are not involved (17).

Currently, the only example of a bacterial outer membrane protein that interacts with the plasma membrane of nonphagocytic mammalian cells to induce endocytic bacterial uptake is the "invasin" product of the *Yersinia pseudotuberculosis inv* gene (63, 65). Although *Shigella* invasion plasmid genes exhibit no significant homology with the *inv* gene, invasin can be considered a conceptual model for putative plasmid-encoded *Shigella* invasion determinants such as IpaBCD. Invasin is a 103-kDa protein that binds to the β_1 subunit of the integrin superfamily of cell adhesin receptors (64). MAbs recognizing either invasin or integrin inhibit the invasion of HEp-2 cells by an *E. coli* K-12 strain expressing the cloned *inv* (invasin) gene (64, 80). Carboxy-terminal fragments consisting of the last 192 amino acids of invasin retain binding activity for HEp-2 cells, and these fragments confer the invasive phenotype when expressed in *E. coli* K-12 (80). The binding of the carboxy-terminal region of invasin to host cell integrin is accompanied by the polymerization of actin microfilaments, and this cytoskeletal communication with integrin is presumably mediated through the talin or fibulin accessory proteins that cross-link both actin and integrins (64). The cytoplasmic signal that induces actin polymerization in response to the binding of invasin is unknown.

Although invasin may serve as a paradigm in the search for a *Shigella* invasion determinant, the invasive phenotype expressed by *Y. pseudotuberculosis*, or by invasive *E. coli* K-12 hybrids expressing the *inv* gene, is fundamentally different from that of shigellae. After invasin-mediated bacterial endocytosis, the intracellular organisms remain within intact endocytic vacuoles (65). In contrast, ingested shigellae rapidly lyse endocytic vacuoles and multiply freely within the cytoplasm of infected mammalian cells (52, 105, 121,

138). The latter phenomenon is apparently mediated by the *Shigella* invasion plasmid because it occurs in HeLa cells that have ingested either plasmid-carrying minicells (55) (Fig. 1) or plasmid-carrying *E. coli* K-12 (121). The lysis of endocytic vacuoles is correlated with the ability to lyse sheep erythrocytes, but this hemolytic assay requires bacterial contact with the erythrocyte membrane (121). The invasive phenotype cannot be differentiated from this hemolytic activity (5, 69), and these phenomena are probably manifestations of binding of the same plasmid-encoded determinant to plasma or erythrocyte membrane receptors. For epithelial cells, this binding induces bacterial endocytosis and eventually causes the lysis of endocytic vacuoles, whereas erythrocytes are lysed from without because they cannot ingest the attached organisms.

Concomitant with the lysis of endocytic vacuoles by invasive *S. flexneri* or *E. coli* K-12 carrying the *Shigella* invasion plasmid, host cell protein synthesis ceases (18, 50, 120) and intracellular ATP levels decrease sharply, reflecting the arrest of both mitochondrial respiration and fermentative metabolic pathways (120). In J774 macrophages that have ingested a noninvasive *S. flexneri* mutant that does not lyse endocytic vacuoles, the inhibition of host cell respiration, fermentation, and protein synthesis is much less pronounced. Although host cell protein synthesis is inhibited by the presence of invasive shigellae in the cytoplasm, transport of amino acids continues in infected cells, and these nutrients are incorporated into the growing bacterial cells (50).

Once the lysis of endocytic vacuoles has occurred, shigellae elicit the localized deposition of F actin in a polar orientation (8). As a result, the organisms begin to move through the cytoplasm of the host cell, leaving a trail of F actin (8), and they infect contiguous cells (79, 87, 105). This process requires bacterial protein synthesis (102), and the plasmid-encoded 120-kDa VirG (IcsA) protein is probably the mediating determinant (8, 79, 87, 105). Another example of actin-mediated intracellular bacterial mobility is found in cultured cells infected with *Listeria monocytogenes*. This gram-positive organism also induces the localized cytoplasmic deposition of F actin in a tail formation that is associated with intracellular bacterial mobility and intercellular bacterial spread (94, 139).

The ability of invasive shigellae to lyse endocytic vacuoles and to spread to contiguous epithelial cells takes on added potential significance in view of histological analysis of rabbit ileal loops showing that, during the initial stages of infection, *S. flexneri* preferentially invade and multiply within M cells that overlie the Peyer patch lymphoid follicles (148). These data suggest that shigellae destroy the intestinal mucosa by lateral spread to the villous epithelium from lymphoid follicles. Since rabbits are not a natural host for shigellae, it could be argued that the villous brush border in rabbits lacks receptors for human enteroinvasive pathogens. However, results of recent studies of rhesus monkeys infected with an *icsA* (*virG*) deletion mutant of *S. flexneri* suggest that the follicle-associated epithelium is also the primary portal of invasion in the primate colon (114a). Endoscopic and histopathological analysis of the colonic mucosa of monkeys infected with this nonspreading mutant revealed small abscesses and bloody ulcerations over lymphoid nodules. Although the general architecture of the inflamed tissue was not typical of a Peyer patch, it is probable that M cells are enriched in these mucosal areas.

Results of similar endoscopic and histopathological analysis of monkey colon 3 days after infection with wild-type *S. flexneri* strains do not suggest a proclivity for infection of follicle-associated epithelium (68), but rapid spread of organisms from M cells to contiguous epithelial cells may obscure the origin of the observed foci of infection. It should be noted that monkeys are relatively resistant to *Shigella* infection compared with humans, and this resistance may also reflect a limited uptake of shigellae by colonic enterocytes. Although it cannot be assumed at this time that these organisms are taken up predominantly by M cells in the human colon, data suggesting that this is case in animal models should stimulate additional research to test this provocative hypothesis in humans.

Conclusion

In a historical perspective, the characterization of the pathogenic mechanism of shigellosis and the discovery of the *Shigella* invasion plasmid must be considered milestones of microbiological research. The next milestone may be the characterization of plasmid and chromosomal gene products in biochemical terms that explain the cell biology of *Shigella* infection. Success in this and in other basic research endeavors should lead to a greater understanding of the biologic relationships of eucaryotic and procaryotic cells, and possibly to vaccines that protect against *Shigella* infections.

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